Single-molecule studies of DNA replication

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Chapter 7: Summary & future prospects

7.1 Introduction

For centuries, people have been wondering how traits are passed on from parents to children. Hippocrates (ca. 460-370 B.C.) and Aristotle (384-322 B.C.) already postulated their thoughts about inheritance. However, it would still take until 1865 before the Czech monk Johann Gregor Mendel experimentally proved the inheritance of traits and established a set of fundamental laws regarding heredity. His results and laws, nevertheless, did not provide information on what physically linked parents and their offspring. Several scientists attempted to unravel this mystery and found that strands of nucleotides, now known as DNA molecules, serve as storage of all our genetic information. In 1953, the structure of DNA was finally proposed by Watson and Crick as being two strands that twist about each other and together form a helix. Importantly, the Watson and Crick DNA model proposed for the DNA to have asymmetric ends, now called the 5' and 3' ends. The 5' end of one strand is paired with the 3' end of the complementary strand resulting in the two DNA strands running in opposite directions.

To successfully transmit hereditary traits from a parent to a daughter cell, transfer of a DNA molecule between those cells is required. To allow transfer of parental DNA to two daughter cells, the parental DNA needs to be converted into two exact duplicates. The process of DNA copying is called DNA replication and is performed by cooperation of several proteins, the DNA replication proteins. Two of the central DNA replication proteins are the helicase and the DNA polymerase. The helicase opens up the DNA helix, providing two single DNA strands that function as a template for the polymerases to synthesize new DNA. Two DNA polymerases synthesize new DNA on the two single DNA strands, one on each strand. These proteins incorporate incoming nucleotides on the single-stranded DNA templates, to convert the single strands into double-stranded DNA. However, the polymerases can only synthesize DNA in a 5' to 3' direction. Since the chemical structure of the DNA strands is antiparallel, this unidirectionality has the important consequence that the two DNA polymerases in the replisome replicate the DNA in opposite directions. As a result, one polymerase follows the helicase and replicates the DNA continuously on what is called the leading strand, whereas the other polymerase, on the lagging strand, has to replicate in a direction opposite to that of the helicase and synthesize the DNA discontinuously, resulting in so-called Okazaki fragments.

To initiate the synthesis of a stretch of DNA, DNA polymerases require a priming of the polymerization reaction by a short RNA primer. Such a primer is synthesized by a separate enzymatic entity, the primase. The synthesis of an Okazaki fragment by the lagging-strand polymerase needs to be preceded by the synthesis of a primer and the recruitment of the polymerase to initiate extension of this primer. Thus, the asymmetry of the DNA has a significant impact on the process of DNA replication and imposes a very tight cooperation of the DNA replication proteins within the replisome!

To allow the lagging-strand polymerase to travel along with the rest of the proteins in the replisome while synthesizing DNA in a direction opposite to the movement of the replication complex, the lagging strand forms a loop, which is called the replication loop. In addition, the helicase can move forward while a primer is being synthesized, in the reverse direction, by the attached primer, which can give rise to a priming loop. The relationship and timing between the development of replication and priming loops and thereby the dynamics of lagging-strand synthesis is poorly understood.

Although the structure and working mechanisms of individual DNA replication proteins are mostly known, the dynamic interactions and exchange mechanisms of DNA proteins within the replisome remain elusive. My PhD work has aimed to contribute to the understanding of the dynamic behavior of lagging-strand polymerases and lagging-strand loops during the process of DNA replication.

7.2 Contributions from this thesis

By performing experiments on the DNA replication machinery of the T7 bacteriophage, which is a virus that can only infect bacteria, the dynamics of lagging-strand polymerases and lagging-strand synthesis was studied. A detailed technical description on how these experiments were carried out is provided in chapter 2. The T7 replisome offers an ideal model system since it can be reconstituted *in vitro* by only four proteins while it maintians the same working principles as higher organisms (26,27). In our experiments, the T7 replication proteins self-assemble into a replisome on a DNA template immobilized on a coverslip. The subsequent addition of proper buffer components enables the replisome to start replication of the DNA template. DNA replication by the replisome can be monitored by fluorescence imaging of either a stained DNA template or the fluorescently labeled replication proteins. The visualization of the duplication of individual DNA templates and the activity of single DNA replication proteins has provided new insights on hitherto unknown working mechanisms of DNA replication proteins.

For example, our work has shown that the lagging-strand polymerases display more dynamic behavior than thought before. Lagging-strand polymerases were shown to frequently exchange with other polymerases in close proximity, implying a hitherto

unknown switching mechanism. Chapter 3 describes our ideas regarding the cause and implications of such an exchange mechanism on protein assembles. The T7 helicase offers two different binding interactions, with different binding affinities, for the T7 DNA polymerases, which can facilitate an exchange mechanism. The DNA-synthesizing polymerases bind the helicase with high affinity while a weaker electrostatic binding tethers additional polymerases to the helicase. On the one hand, these two interactions provide stable binding of polymerases during DNA replication and, on the other, allow binding of additional polymerases that function as substitutes for the DNA synthesizing polymerases. This bimodal mechanism allows for rapid polymerase exchange in the presence of additional polymerases, but also their prolonged binding in the absence of competing polymerases. The presence of such a polymerase exchange mechanism on the discontinuously synthesized lagging strand raises the question whether there are other dynamically exchanging proteins within the replisome, for example the single-stranded DNA-binding proteins.

Experimental evidence of the highly dynamic exchange of the T7 polymerases within the replisome is presented in chapter 4. By making use of a single-molecule fluorescence readout, labeled polymerases within the replisome were visualized while DNA was replicated. This readout allowed for the determination of the kinetics of lagging-strand polymerases and the stoichiometry of polymerases at the replication fork. Our data suggest that the lagging-strand polymerases dissociate from the replisome after the synthesis of only a few Okazaki fragments. In addition, two or more polymerases were found to be present within the replisome during DNA replication activity. As a result, a highly dynamic picture of the replisome emerges with lagging-strand polymerases being frequently exchanged, after synthesis of only a few Okazaki fragments, by readily available replisome-bound polymerases that are continuously replenished from solution. These new findings suggest an adjustment of the original textbook pictures (24) and lead us to wonder whether dynamic polymerase exchange is preserved among replisomes of other organisms as well.

Further insight into the dynamics of lagging-strand synthesis and the function of lagging-strand polymerases that are released by the replisome is provided in chapter 5. An innovative DNA template was used that was coupled to two beads (one on each end) and surface immobilized in the middle. By tracking the position of both beads in time, the coordination between leading- and lagging-strand synthesis was monitored. Our data suggest the presence of both ss-ds and ss loops, where unexpectedly, most loop growth events occur only during priming. Additional time-lapse fluorescence imaging showed that most polymerases were not recycled but instead were released from the replisome, presumably to complete synthesis of unfinished Okazaki fragments. Individual replication events revealed numerous reaction cycle types and pausing events, supporting the notion that, while an array of critical interactions and regulatory circuits guide replisome function, a multitude

of kinetic pathways are utilized. Further research on the kinetics of polymerases that are loaded on the lagging strand will contribute to understanding of the dynamics of lagging-strand synthesis and the mechanism of coordination of DNA replication of both DNA strands by the replisome.

Over the past years, single-molecule fluorescence experiments have provided insights into dynamic properties of biomolecular processes. However, the visualization of individual fluorescently labeled proteins at high concentrations has been technically difficult. As a consequence, single-molecule fluorescence experiments are usually employed *in vitro* at labeled protein concentrations far below the *in vivo* concentrations, while their aim is to study biomolecular interactions in living organisms. Chapter 6 describes a new approach that enables the observation of individual labeled proteins while the concentration of fluorescent molecules in solution is very high. This new approach employs the ability to regulate the excited state of the fluorophores by changing the experimental conditions. Fluorophores can chemically be brought into a dark non-fluorescent state and subsequently recover their fluorescent state by excitation with light of a specific wavelength. This excitation is mostly emitted by a laser, but can also be sent transferred from another fluorescent molecule. However, the excitation of the fluorophore by another fluorophore can only happen over very short distances. The new technique (described in chapter 6) utilizes the short-distance fluorescence activation of fluorophores and as such is named LADye (Local Activation of Dye).

Our experiments showed that excitation of a stained-DNA template caused the switching of a proximal fluorophore to a fluorescent state. Subsequently, a fluorophore was coupled to a DNA-binding protein. Excitation of the DNA caused the activation of the fluorescence of only those labeled proteins that interacted with the DNA template while the unbound proteins in solution remained dark. This experimental design allowed us to observe the binding and sliding behavior of two different DNA-binding proteins, IFI16 and pVIc-AVP, on DNA in the presence of a high concentration of fluorophores in solution. However, LADye is presumably not limited to a DNA-based system, but can be a generally applicable method to study biomolecular interactions. As such, the LADye approach will be useful for future studies on biomolecular processes within physiological crowding environment as can found in cells.